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Cutting Edge: IL-1 Receptor-Associated Kinase 4 Structures Reveal Novel Features and Multiple Conformations

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IL-1R-associated kinase (IRAK)4 plays a central role in innate and adaptive immunity, and is a crucial component in IL-1/TLR signaling. We have determined the crystal structures of the apo and ligand-bound forms of human IRAK4 kinase domain. These structures reveal several features that provide opportunities for the design of selective IRAK4 inhibitors. The N-terminal lobe of the IRAK4 kinase domain is structurally distinctive due to a loop insertion after an extended N-terminal helix. The gatekeeper residue is a tyrosine, a unique feature of the IRAK family. The IRAK4 structures also provide insights into the regulation of its activity. In the apo structure, two conformations coexist, differing in the relative orientation of the two kinase lobes and the position of helix C. In the presence of an ATP analog only one conformation is observed, indicating that this is the active conformation. The Journal of Immunology, 2007, 178: 2641–2645.

Materials and Methods

Protein production

To determine the most suitable boundary of the IRAK4 kinase domain for molecular structure determination, sequential 1-aa N-terminal truncations (residues 150–165) and 3-aa C-terminal truncations (residues 417–453) were made from IRAK4 cDNA. Truncated IRAK4 constructs were generated by overlap-extension PCR in which (from the N terminus) a 6-His tag, TEV protease spacer, and TEV cleavage site were encoded. PCR products were subcloned into the baculovirus transfer vector pVL1392 (BD Biosciences), and molecular structure determination, sequential 1-aa N-terminal truncations (residues 150–165) and 3-aa C-terminal truncations (residues 417–453) were made from IRAK4 cDNA. Truncated IRAK4 constructs were generated by overlap-extension PCR in which (from the N terminus) a 6-His tag, TEV protease spacer, and TEV cleavage site were encoded. PCR products were subcloned into the baculovirus transfer vector pVL1392 (BD Biosciences), and DNA sequence was verified. Correct constructs were cotransfected into Sf9 cells, and the produced virus was plaque-purified to generate clonal populations. Tittered, plaque-purified virus was used to determine that a multiplicity of infection of 0.3 with a harvest time of 72 h was optimal for protein expression. An N-terminal truncation in which amino acid residues 160 through 460 of IRAK4 are expressed was one candidate identified to possess good expression and purification properties.

For large-scale purification of IRAK4 kinase domain, pellets from 10 L of infected Sf9 cell was resuspended in 600 ml of lysis buffer (50 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM 2-ME, 12 Complete protease inhibitor tablets (Roche Applied Science), 5 mg of DNase per 100 mg of cells). Cells were then lysed with a microfluidizer (Microfluidics) at <9,000 psi on ice. The lysate was centrifuged at 25,000 × g, and the supernatant was applied to a 50-ml Talon Superflow metal affinity column (BD Biosciences) pre-equilibrated in...
column buffer A (50 mM HEPES (pH 7.5), 300 mM NaCl, 10% (v/v) glycerol, 10 mM 2-ME, and 20 mM imidazole). The bound protein was eluted with buffer B (buffer A with 100 mM imidazole (pH 7.5)) and concentrated to 5 mg/ml. The 6-His tag was cleaved overnight at 4°C with AcTEV protease (Invitrogen Life Technologies) in 0.3% (v/v) n-octyl-β-D-glucopyranoside, <20 mM imidazole, 1× stock buffer, 1 mM DTT, and 2,000 U AcTEV. The reaction mixture was passed over a 5-ml Ni-HP column (GE Healthcare) pre-equilibrated in buffer A, and the flow-through was concentrated to 5–10 ml with an Amicon Ultra-15 concentrator (Millipore). IRAK4 kinase domain was finally purified on a Superdex 200 26/60 column (GE Healthcare) and concentrated to 13 mg/ml.

**Enzymatic assay**

IRAK4 kinase domain activity was measured by phosphorylation of the IRAK1 peptide substrate KKARFSRFAGSSPSQSSMVAR (Anaspec) using [γ-32P]ATP (Amersham). The enzyme reaction was conducted in 25 mM HEPES (pH 7.5), 2 mM MgCl2, 150 mM NaCl, 20 mM MgCl2, 0.001% Tween 20, and 0.1% bovine γ-globulin (Sigma-Aldrich). For ATP Km determinations, initial rates of enzymatic reactions were measured using 4 nM enzyme in the presence of 4.5 mM peptide substrate and 0.1–6.4 mM ATP. The inhibition potency of staurosporine was determined by measuring the activity of 2 nM IRAK4 kinase domain in the absence and presence of 10 inhibitor concentrations at 0.25 Km concentrations of both ATP and the peptide substrate.

**Crystallization, data collection, and processing**

The IRAK4 kinase domain at a concentration of 13 mg/ml (50 mM HEPES (pH 7.5), 10% (v/v) glycerol, 300 mM NaCl, 10 mM 2-ME, and 0.02% (v/v) octyl-β-D-glucopyranoside) was incubated for 2 h at 4°C either with 1 mM staurosporine (Sigma-Aldrich) and 5% (v/v) DMSO or with 12 mM AMPPNP (Sigma-Aldrich). The protein was crystallized by vapor diffusion in hanging drops at 20°C. A total of 0.7 μl of protein solution was mixed with 0.7 μl of 2.3 M sodium malonate, 100 mM sodium acetate (pH 5.0), and 10 mM DTT resulting in a final pH of 7.0. Crystals of space group C2 (a = 147Å, b = 139Å, c = 89Å, β = 124°) appeared within 3–10 days and grew to a maximum size of 300 × 300 × 200 μm³. Because IRAK4 crystals grown after incubation with AMPPNP showed no ligand density at the ATP binding site, they were soaked with saturated solution of AMPPNP for 6 h.

X-ray diffraction data of apo and AMPPNP-bound IRAK4 kinase domain was collected at the synchrotron beam line 9-1 of the Stanford Synchrotron Radiation Laboratory (Palo Alto, CA) using an ADSC Quantum 310 CCD detector. Data of the IRAK4-staurosporine complex was collected at beam line 16.0.2 at the Advanced Light Source (Berkeley, CA) using an ADSC Quantum 210 CCD detector. All diffraction images were processed with DENZO and the intensities were scaled with SCALEPACK (7).

**Molecular replacement, structure refinement, and model building**

The search model for molecular replacement is based on the crystal structure of the proto-oncogene serine/threonine-protein kinase B-RAF (Ref. 8; Protein Data Bank accession no. 1UWH). Regions of very low sequence similarity were deleted, and the remaining amino acids were exchanged to the IRAK4 sequence using the program XSAE (C. Broger, F. Hofmann-La Roche, Basel, Switzerland). Molecular replacement was performed with the program Phaser (9). The solution found was an IRAK4 tetramer in space group C2. The model was refined against the experimental data, and electron density maps were calculated using REFMACS (10). The structure model was built with the graphics software MOLOC (11) and validated with PROCHECK (12). Illustrations of the final IRAK4 crystal structures were created using PYMOL (Delano Scientific).

**Results and Discussion**

**Overall structure and ligand binding**

We have determined the x-ray crystal structures of IRAK4 kinase domain in its nonliganded form as well as complexed with the natural microbial alkaloid staurosporine and the nonhydrolysable ATP analog AMPPNP (Table I). The IRAK4 kinase domain protein selected for crystallization is catalytically active (ATP Km = 650 μM, substrate Km = 1100 μM, kcat = 420 min⁻¹). IRAK4 kinase domain crystals contain four protein chains in the asymmetric unit. Although several structural differences are observed between the four proteins in the asymmetric unit and between the different ligand complexes at the level of individual amino acids and positioning of secondary structure elements, their overall architectures are conserved. IRAK4 kinase domain possesses the typical two-lobe structure. The N-terminal lobe consists of a five-stranded antiparallel β-sheet, helix C, and a nonprotopotypic N-terminal α-helix (helix B) that packs against the β-sheet (Fig. 1). In addition, a unique loop is present in the IRAK4 kinase domain between the N-terminal helix and the first β-strand, which is significantly shortened by this insertion. The C-terminal kinase lobe of IRAK4 is comprised of five larger and two shorter α-helices, one β-hairpin, the activation loop, and several additional loop structures. The N- and C-terminal lobes are connected by the so-called hinge region, which partially defines the binding site for ATP and ATP-competitive kinase inhibitors.

Recently, three autophosphorylation sites in the activation loop of the IRAK-4 kinase domain have been identified: p-T342, p-T345, and p-S346 (13). Mutations at these positions reduce IRAK-4 kinase activity significantly. We have independently identified the same three phosphoresidues in electron density maps of insect cell-expressed IRAK4 kinase domain (Fig. 2a). p-T345 represents the prototypical phosphoside required for the activation of many protein kinases.

**FIGURE 1.** Staurosporine-bound IRAK4 kinase domain. Green, Gly-rich loop; cyan, helix C; magenta, activation loop; brown, helix B; red, loop unique to IRAK4; yellow, staurosporine. Amino acids not resolved in the electron density maps are outlined as dots.
droxyl groups and the protein. It is possible that this missing hydrogen bond is observed between either of the two ribose backbone amine and the V263 backbone carbonyl group. No position with the adenine hydrogen bonding to the M265 backbone amine. In addition, the phosphate forms water-mediated hydrogen bonds with the backbone carbonyl groups of P184/I185, respectively (Fig. 2B). The lipophilic bulk of staurosporine makes numerous hydrophobic and van der Waals’ interactions with the predominantly lipophilic ATP-binding pocket of IRAK4. In addition, one of the two staurosporine phenyl rings is forming an offset stacked aromatic interaction with the phenyl ring of the hinge residue Y264, while the other phenyl ring is forming an edge-to-face aromatic interaction with the phenyl ring of the gatekeeper residue Y262.

Electron density maps of the crystal structure of IRAK4 kinase domain. Because the electron density of the AMPPNP phosphates is of limited quality, their interactions with the protein and solvent molecules could not be resolved unambiguously.

Unique N-terminal extension and gatekeeper residue

The primary challenge in designing therapeutic kinase inhibitors is to achieve the desired selectivity, because off-target kinase inhibition may result in adverse effects. Therefore, identification of unique features for a kinase drug target is critical for successful drug design. The N-terminal kinase lobe of IRAK4 has two defining features not seen in previously reported kinase structures: a unique N-terminal extension and tyrosine as gatekeeper residue.

In addition to the five-stranded antiparallel β-sheet and helix C, the N-terminal kinase lobe of IRAK4 contains an N-terminal extension of so far unknown function that is unique to IRAK4 as judged by a sequence alignment of 518 human kinases, including the other IRAK family members (15). It starts with an amphiphilic α-helix (helix B, aas 169–176) that makes extensive hydrophobic interactions with lipophilic side chains of amino acids forming the β-sheet (Fig. 1). This α-helix is followed by three motifs that have been described as helix-capping elements: 1) an ST motif (Ref. 16; aas 177–181), which is characterized by hydrogen bonds from the T177 hydroxyl group to the F180 backbone amine and from the T177 backbone carbonyl group to the D181 backbone amine; 2) an ASX motif (17; aas 181–183) defined by the hydrogen bond between the D181 side chain carbonyl group and the R183 backbone amine; and 3) a highly solvent-exposed Schellman loop (Ref. 18; aas 184–189) characterized by hydrogen bonds between the backbone amines of G188/G189 and the backbone carbonyl groups of P184/I185, respectively (Fig. 3). C-terminal to the IRAK4 loop continues the prototypic Gly-rich loop, which is formed by the first two strands of the five-stranded β-sheet. The Schellman loop, in particular I185, is adjacent to the ATP binding site, which makes it an ideal interaction partner for IRAK4 selective kinase inhibitors.

**FIGURE 2.** F,C,F,F electron density omit maps contoured at 3σ. Selected hydrogen bonds are indicated as dashes. Activation loop residues (A), staurosporine (B), and AMPPNP (C) are shown in yellow, and contacting amino acids are shown in gray.

**FIGURE 3.** N-terminal region unique to IRAK4. Hydrogen bonds that characterize the ST, ASX, and Schellman motifs are indicated as dashes.
Immediately N-terminal to the hinge loop (aas 263–268) is the so-called gatekeeper residue, Y262 in IRAK4. The size and flexibility of the side chain of this residue generally determines whether an additional lipophilic subpocket, referred to as the “back pocket,” is present adjacent to the ATP binding site (19). The most abundant gatekeeper residue in kinases is methionine, followed by leucine, threonine, and phenylalanine. As judged by primary sequence alignments of human kinase domains, tyrosine occupies the gatekeeper position only in the four members of the IRAK family. The IRAK4 crystal structures reported in this study are the first structural representatives for kinases with tyrosine as a gatekeeper residue. The bulky side chain of the gatekeeper Y262 is oriented toward the conserved E233 forming a hydrogen bond with the glutamate side chain (Fig. 4). Therefore, the back pocket of IRAK4 is blocked by the gatekeeper tyrosine and not accessible for ligands. However, ligand interactions with the hydroxyl group of the gatekeeper residue Y262 should be considered in the design of IRAK family selective inhibitors.

Two kinase domain conformations and implications on IRAK4 regulation

Many kinases are activated by phosphorylation of activation loop residues, either by upstream kinases or autokatallytically. It has also been shown for several kinases that a hinge movement of helix C away from the ATP binding site is involved in switching thses enzymes from their catalytically active to an inactive state (14). This process results in disruption of an essential salt bridge between an invariant lysine on strand 3 of the antiparallel β-sheet and an invariant glutamate on helix C. Several different mechanisms have been described that trigger the position of helix C and therefore the activation state of the kinase. Examples include the intermolecular interaction between helix C of cyclin-dependent kinase and cyclin (20, 21), and the intramolecular interaction between helix C of c-Src and the c-Src SH3 domain (22, 23).

The IRAK4 apo-form crystal structure shows two distinct protein conformations in the tetramer comprising the crystallographic asymmetric unit. These conformations differ significantly in their relative orientation between their N- and C-terminal lobes. The regions of the protein primarily affected by the lobe rotation of IRAK4 are the Gly-rich loop, the unique N-terminal extension, and helix C (Fig. 4). The overall effect of this rotation on helix C is a similar hinge movement as described above for other kinases. We therefore refer to the two IRAK4 kinase domain conformations as “helix C-in” and “helix C-out.” The positioning of helix C in IRAK4 has the opposite effect on the lysine-glutamate salt bridge (Fig. 4) compared with other kinases. The K213-E233 interaction in IRAK4 is only present in the helix C-out position. In the helix C-in position, the K213 side chain is disordered and the carboxyl group of E233 forms a hydrogen bond with the backbone amine of F350, which is part of the conserved DFG sequence that marks the N-terminal end of the activation loop.

In the IRAK4-AMPPNP structure, all four kinase domains present in the crystallographic asymmetric unit adopt the helix C-in conformation. As observed in the apo structure, in this conformation the K213-E233 salt bridge is not formed. Presumably, in the absence of ATP analog both kinase domain conformations coexist in solution. Binding of AMPPNP between the N- and C-terminal kinase lobes shifts this equilibrium to the helix C-in conformation. The presence of AMPPNP at the ATP binding site was achieved by soaking AMPPNP into apo crystals, therefore the transition from the helix C-out to the helix C-in conformation can occur in the crystal lattice. The fact that AMPPNP binds only to the helix C-in conformation of IRAK4 suggests that this represents the active form of the kinase, despite the missing K213-E233 salt bridge (Fig. 4). It is possible that the presence of the hydroxyl group of the unique Y262 gatekeeper side chain affects the local hydrogen bond pattern requirements for nucleotide binding.

The mechanism of IRAK4 activation in cells is not well understood. In analogy with other kinases, it seems likely that IRAK4 phosphoryl transfer activity is regulated both by activation loop phosphorylation and by switching between the two observed (or more) kinase domain conformations (Fig. 4). In the case of IRAK4, this switch could be triggered by intramolecular interaction between kinase domain and its death domain or by intermolecular interactions with adaptor proteins like MyD88. The crystal structures of IRAK4 kinase domain presented in this study form the basis for the design of experiments that decipher these interactions, and therefore how IRAK4 mediates IL-1/TLR signaling.

Note Added in Proof. After submission of this manuscript, Wang et al. (24) published the crystal structure of IRAK4 kinase domain complexed with staurosporine and a benzimidazole inhibitor.

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Disclosures

The authors have no financial conflict of interest.

References


